



## Deciphering the Upper Respiratory Tract Microbiota Complexity in Healthy Calves and Calves Developing Respiratory Disease Using Shotgun Metagenomics

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**Interpretive summary: Metagenomics of bovine respiratory disease**

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We evaluated the microbiota of the upper respiratory tract of healthy Holstein calves and calves that developed bovine respiratory disease using shotgun metagenomics. Deep nasopharyngeal swabs were performed on 16 Holstein heifer calves at 14 and 28 days of life. Samples were dominated by five predominant phyla: *Proteobacteria*, *Firmicutes*, *Actinobacteria*, *Bacteroidetes* and *Tenericutes*. At the genus level, differences between groups were observed for *Pseudomonas* spp. *Moraxella catarrhalis* was detected in all groups of samples. The resistance to antibiotics and toxic compounds profiling analysis revealed differences in cobalt-zinc-cadmium resistance especially between BRD and healthy calves at their 14<sup>th</sup> day of life.

**METAGENOMICS OF BOVINE RESPIRATORY DISEASE**

**Deciphering the Upper Respiratory Tract Microbiota Complexity in Healthy Calves and Calves Developing Respiratory Disease Using Shotgun Metagenomics**

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ABSTRACT

Bovine Respiratory Disease (BRD) is a multi-factorial disorder that remains responsible for severe economic losses within dairy and feedlot herds. Advances in next generation sequencing allow the characterization of microbial communities present in clinical samples, including non-culturable bacteria. Our aim here was to evaluate the microbiota of the upper respiratory tract of healthy calves and calves developing BRD using whole genome sequencing (shotgun metagenomics). Deep nasopharyngeal swabs were performed on sixteen Holstein heifer calves (ten healthy and six that were diagnosed with BRD during the study) at 14 and 28 days of life in one dairy herd located near Ithaca, New York. Total DNA was extracted and whole genome sequencing was performed using the MiSeq Illumina platform. Samples were dominated by five predominant phyla: *Proteobacteria*, *Firmicutes*, *Actinobacteria*, *Bacteroidetes* and *Tenericutes*. At the genus level, differences between groups were observed for *Pseudomonas* spp. At the species level, *Mannheimia haemolytica* was the most abundant bacterium detected. Significant differences between different groups of calves were detected in the relative abundance of *Pseudomonas fluorescens*. *Pasteurella multocida* was amongst the 20 most abundant species. Additionally, *Moraxella catarrhalis*, commonly associated with pneumonia in humans, was detected in all groups of calves. The resistance to antibiotics and compounds profiling analysis revealed differences in cobalt-zinc-cadmium resistance. Further research that will elucidate the role of *Moraxella catarrhalis* in BRD is warranted. Cobalt-Zinc-Cadmium resistant genes observed mostly in BRD calves might be associated with difficulties in the treatment using antibiotics.

**Key words:** Bovine Respiratory Disease, Whole Genome Sequencing, Calf

## INTRODUCTION

Bovine Respiratory Disease (**BRD**) is a multi-factorial disorder (Gershwin et al., 2015) that remains responsible for severe economic losses in dairy and feedlot herds, due to its high morbidity and mortality rates (USDA, 2013). Furthermore, BRD has substantial long-term consequences on performance by negatively impacting growth, reproductive performance, and longevity (Waltner-Toews et al., 1986; Virtala et al., 1996a,b; Cernicchiaro et al., 2013). It has been estimated that 7% of the total production cost in feedlots in the United States is related to BRD (Griffin, 1997).

The upper respiratory tract of animals normally harbors a variety of microorganisms that coexist in a harmonious state (Bosch et al., 2013). However, certain events can lead to imbalances in this microbiome and eventually to respiratory disease. In cattle, viral infections, stress (i.e. transportation and mixing), and environmental conditions (i.e. stocking density, climate, and temperature) are important predisposing factors to this imbalance, leading to respiratory tract bacterial infections facilitated by the host's immune suppression (Griffin et al., 2010). The most commonly described bacteria in BRD are *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni* and *Mycoplasma* spp., particularly *Mycoplasma bovis* (Aebi et al., 2015; Holman et al., 2015a). These species are considered part of the upper respiratory tract microbiome. Similarly, humans' upper respiratory tract serves as a reservoir of commensal bacteria such as *Haemophilus influenza*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Moraxella catarrhalis* which can in certain cases invade the lungs and cause pneumonia (Bosch et al., 2013).

Culture-dependent techniques are often used as a diagnostic method to determine microorganisms associated with respiratory disease. However, certain limitations are associated with these techniques, the main one being that several important microbes are difficult if not impossible to culture in the laboratory (Sharpton, 2014). Advances in next generation sequencing have allowed in depth investigation of clinical samples' microbiomes, determining its taxonomic

composition including unculturable species. Whole genome sequencing (shotgun metagenomics), differently from 16S metataxonomics, provides with taxonomic descriptions of microbial communities, but at the same time creates important information regarding gene prediction, protein diversity and functional profile (Sharpton, 2014).

Studies using 16S metataxonomics for the evaluation of the nasal, nasopharyngeal and lung microbiota in feedlot cattle have already been published (Holman et al., 2015b; Timsit et al., 2016). However, and to the best of our knowledge, shotgun metagenomics has not been used yet to investigate BRD. Therefore, our aim was to characterize the microbiota of the upper respiratory tract of healthy calves and calves developing BRD using whole genome sequencing, and identify bacteria that may be related to the development of BRD and describe their functional profile.

**MATERIAL AND METHODS**

The present study was conducted at the Department of Population Medicine and Diagnostic Science, College of Veterinary Medicine, Cornell University, New York, from August 2015 until January 2016. Sixteen Holstein heifer calves were selected from a larger cohort of 174 animals that were included in a different experiment (Lima et al., 2016). This larger scale study was conducted on a commercial dairy farm located near Ithaca, New York that at the time of the study had around 7000 animals in total (3,500 lactating Holsteins cows), and approximately 600 pre-weaning calves that were raised in groups of 25 animals per pen. All procedures were carried out in agreement with the guidelines of the Institutional Animal Care and use Committee of Cornell University (Protocol number: 2013-0076).

***Animals and facilities***

Pregnant cows at stage 1 or 2 of parturition were transferred from the close-up free-stall barn into two maternity pens (400 m<sup>2</sup> deep-bedded pens). After parturition, calves were removed from

the maternity pen and placed into a newborn pen bedded with dry sawdust and heated with heating lamps during the winter months. Feeding and pen characteristics were described by Lima et al., (2016). Calves used in this study were allocated in 9 different pens (25 calves per pen) and all calves remained in the same pen from day one of life until fully weaned (approximately 65 days of life). The age range of calves within a pen was not greater than 3 days.

### ***Case definition***

BRD was initially defined when two or more of the following clinical signs were detected in a calf: cough, rectal temperature  $> 39.5^{\circ}\text{C}$ , respiratory rate  $> 40$  breaths/min, increased cranioventral lung sounds or wheezes. Two dedicated farm employees with over 10 years of experience and trained by Cornell University veterinarians (Ambulatory and Production Medicine Department), were responsible for overseeing the calf facility and making the initial detection of BRD. An experienced veterinarian member of our research team performed a second confirmatory physical examination. Calves diagnosed with BRD were treated according to standard farm protocol (Resflor Gold, Merck Animal Health). Treatment was administered at the day of the diagnosis.

### ***Deep nasopharyngeal swab collection***

As part of this different larger scale observational study deep nasopharyngeal (DNP) swabs were collected from a cohort of 174 Holstein heifer calves on days 3, 14, 28, and 35 of their life using a 20-cm DNA-free sterile swab (Puritan Medical Products, Guilford, ME) covered by a thin sterile plastic sheath, as described by Lima et al. (2016). Samples were kept on ice until they were transferred to the laboratory and then stored at  $-20^{\circ}\text{C}$  until further processing. Deep nasopharyngeal swabs collected at 14 and 28 days of life from 10 calves that remained healthy throughout the pre-weaning period (randomly selected among all the calves that remained healthy throughout the study) and 6 calves that showed clinical signs of BRD before weaning (randomly selected among all the calves that showed signs of BRD during the study) were used for the present study.

Bovine respiratory disease was on average diagnosed on the 19<sup>th</sup> day of the calves' life. The earliest day of diagnosis was the 14<sup>th</sup> with the latest being the 26<sup>th</sup>. We therefore had four groups of samples: 10 samples obtained from healthy calves at 14 days of life (healthy-14d); 6 samples obtained at 14 days of life from calves that developed BRD between 14 and 26 days of life (BRD-14d); 10 samples obtained from healthy calves at 28 days of life (healthy-28d); and 6 samples obtained at 28 days of life from calves that developed BRD between 14 and 26 days of life (BRD-28d).

### ***DNA extraction and Whole Genome Sequencing***

Total DNA was extracted from DNP samples by adding 1.5 mL of DNA-free water into microcentrifuge tube containing cotton swabs, followed by mechanic disruption using a Mini-Beader-8 (Biospec Products, Battersville, OK). Swabs were removed from the tubes and the remaining liquid was centrifuged at 13,000 x g for 10 minutes at room temperature. The DNA was extracted from pellets using PowerSoil DNA Isolation Kit (MO BIO Laboratory Inc., Carlsbad, CA) according to the manufacturer's protocol (Lima et al., 2016).

Samples were diluted by adding UltraPure Water (Invitrogen, Waltham, MA) until a concentration of 2.5ng/ $\mu$ L was reached, measured using Qubit (Thermo Fisher Scientific, Waltham, MA) fluorometer. Sample library was prepared using Nextera DNA Sample Preparation Kit (Illumina Inc. San Diego, CA) according to the manufacturer's protocol.

The library for cluster generation and sequencing was prepared according to Illumina's protocol. Briefly, a mixture of 5  $\mu$ L DNA (4nM) and 0.2 N NaOH was vortexed and centrifuged to 280 x g for 1 minute. The mixture was incubated for 5 minutes to allow DNA denaturation. Following DNA denaturation, 990  $\mu$ L of Hybridization Buffer were added to dilute DNA and obtain a 2 nM library. Quality control for cluster generation, sequencing and alignment was provided by adding the PhiX Control - 4 nM (Illumina Inc. San Diego, CA), prepared using 2  $\mu$ L of



10 nM PhiX library and 3  $\mu$ L of 10 mM Tris-Cl pH8.5 with 0.1% Tween 20. Pair-end sequencing was performed using a MiSeq Reagent Kit v3 (600-cycles) in the Illumina MiSeq platform.

### ***Bioinformatics and statistical analysis***

Raw data files were de-multiplexed and converted to fastq files using Casava v.1.8.2 (Illumina, Inc, San Diego, CA). Fastq files were concatenated and uploaded to the Metagenome Rapid Annotation using Subsystem Technology (**MG-RAST**) server to determine relative abundance of the microbiota at the phylum, genus and species levels, and metabolic characteristics prediction. In the MG-RAST pipeline, sequences were subjected to quality control, including dereplication (removal of sequences produced by sequencing artifacts), removal of host-specific species sequences (*Bos taurus*, UMD v3.0), ambiguous base filtering (removal of sequences with >5 ambiguous base pairs) and a length filtering (removal of sequences with a length >2 standard deviations from the mean). To reduce the impact of experimental noise/error, the normalized data option of MG-RAST was used. Normalization was performed by a log<sub>2</sub>-based transformation ( $\log_2(x + 1)$ ) followed by standardization within each sample and linear scaling (across all samples). Non-redundant multi-source protein annotation database (M5NR) was the annotation source used for organism classification. Organism abundance was analyzed using a “Best Hit Classification” approach with a maximum e-value of  $1 \times 10^{-5}$ , minimum identity cutoff of 60%, and a minimum alignment length cut-off of 15. The SEED was the annotation source used for predicted metabolic profiles. The predicted metabolic abundance analysis was performed using “Hierarchical Classification”, with a maximum e-value of  $1 \times 10^{-5}$ , minimum identity of 60%, and a minimum alignment length cut-off of 15.

Normalized abundances of phyla, genera, species and functional profile elements were extracted from MG-RAST and analyzed using ANOVA in conjunction with Tukey test for multiple comparisons using JMP Pro 12 (SAS Institute Inc., NC). Heatmap for phyla distribution were constructed in JMP Pro 12. Results are presented as least square means followed by the standard

error of the mean. Dependent variables in our statistical analyses were the normalized relative abundances of microbial types with the independent variable being the different health status and sampling time (BRD -14d; BRD-28d; Healthy-14d; Healthy-28d). Comparisons were performed in order to identify potential bacteria and functional features that may be related to the development of BRD during the period that calves are more predisposed to it (Virtala et al., 1996). Variables with  $P < 0.05$  were considered statistically different. Variables with  $0.1 < P < 0.05$  were considered as statistical tendencies.

Shotgun sequencing data have been deposited in MG-RAST under the following accession numbers (4678805.3, 4678806.3, 4678807.3, 4678808.3, 4678809.3, 4678810.3, 4678811.3, 4678812.3, 4678813.3, 4678814.3, 4678815.3, 4678816.3, 4678817.3, 4678818.3, 4678819.3, 4678820.3, 4678821.3, 4678822.3, 4678823.3, 4678824.3, 4678825.3, 4678826.3, 4678827.3, 4678828.3, 4678829.3, 4678830.3, 4678831.3, 4678832.3, 4678833.3, 4678834.3, 4678835.3, 4678836.3).

RESULTS

*Sequencing results*

Sequencing of DNP samples generated 21,155,233 sequences (mean number of sequences per sample was: 661,101; median: 638,404; range = 383,190 - 994,172); 2,307,911,887 basepairs (mean: 72,122,246.46; median: 73,160,664; range = 40,628-100,847,895); 310,656 high quality sequences (mean: 9,708; median: 9,330; range = 4,954-16,210), and an average length of 111.1 (median: 115.5; range = 74 - 138).

*Nasopharyngeal microbiota*

In total, 22 phyla were detected; however, five predominant phyla were identified as the most abundant in all groups: *Proteobacteria*, *Firmicutes*, *Actinobacteria*, *Bacteroidetes* and *Tenericutes* (Figure 1). Together, these major phyla comprise 96%, 97%, 95% and 80% of detected phyla of BRD-14d, BRD-28d, healthy-14d and healthy-28d calves, respectively. Within *Proteobacteria*, *Pseudomonas* spp. was the most abundant (11%), from which *Pseudomonas fluorescens* showed the highest abundance. This phylum was mainly constituted of *Pseudomonas* spp., *Psychrobacter* spp., *Mannheimia* spp., *Actinobacillus* spp., *Enhydrobacter* spp., *Escherichia* spp., *Acinetobacter* spp., *Haemophilus* spp. and *Moraxella* spp..

A significant difference in the phyla relative abundance between groups was observed for the phylum *Bacteroidetes* ( $P < 0.05$ ). Comparison of *Bacteroidetes* mean relative abundance between health status and sampling time point revealed significant differences between healthy-28d and healthy-14d samples ( $P < 0.05$ ).

Analysis at the genus level revealed 20 dominating genera (Figure 2). The most important genera related to respiratory diseases, such as *Mycoplasma* spp., *Mannheimia* spp., and *Pasteurella* spp. were detected in all groups (Figure 2), and numeric, although statistically not significant, differences were observed.

Relative abundances of the 20 most abundant bacteria (species level information) detected in DNP samples from healthy calves and calves that developed BRD are shown in Figures 3 and 4. Relative abundance of *Pseudomonas fluorescens* for each different group of calves was  $0.07 \pm 0.04$  (BRD-14d),  $0.002 \pm 0.002$  (BRD-28d),  $0.01 \pm 0.005$  (healthy-14d) and  $0.002 \pm 0.001$  (healthy-28d) ( $P < 0.05$ ). Despite no significant differences being detected between the main bacteria related to pneumonia in calves [*Mannheimia haemolytica* ( $P = 0.60$ ) and *Pasteurella multocida* ( $P = 0.83$ )] and health status, these pathogens were within the 20 most abundant species, unlike *Histophilus somni* and *Mycoplasma bovis* (Figures 3 and 4). *Mannheimia haemolytica* was the most abundant bacterium and its relative abundance was numerically increased in both BRD-28d ( $0.06 \pm 0.03$ ) and

healthy-28d calves ( $0.07 \pm 0.02$ ) comparing to BRD-14d and healthy-14d calves. On the other hand, *Pasteurella multocida* relative abundance was low in all four groups (Figure 4). *Moraxella catarrhalis* was also detected in all groups of calves. Relative abundance of the bacteria most commonly associated to pneumonia (namely *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni*, and *Mycoplasma bovis*) are also presented in Figure 6.

The proportion of calves with a positive detection of these known pathogens is presented in Table 1. *Mannheimia haemolytica* was detected in most of the studied calves (78.12%). On the other hand, lower proportion was observed for *Mycoplasma bovis* (18.75%). *Mycoplasma bovis* was detected in 16.7% of the calves in both BRD groups and in 40% of the healthy-28d calves.

Analysis at the strain level revealed numeric differences between healthy animals and animals that developed BRD for *Mannheimia haemolytica* non-serotype A1 str. PHL213 ( $P = 0.11$ ) (Figure 5). Relative abundance of this specific serotype (expressed as a proportion out of all *Mannheimia haemolytica* serotypes) was increased in BRD-14d calves ( $0.44 \pm 0.14$ ) and BRD-28d ( $0.49 \pm 0.11$ ) compared to healthy-14d ( $0.35 \pm 0.12$ ) and decreased compared to healthy-28d calves ( $0.53 \pm 0.09$ ). *Mannheimia haemolytica* serotype A1 str. PHL213 relative abundances were also increased in BRD-14d calves ( $0.23 \pm 0.07$ ) and BRD-28d ( $0.34 \pm 0.09$ ) compared to healthy-14d ( $0.15 \pm 0.05$ ) and decreased compared to healthy-28d calves ( $0.37 \pm 0.08$ ) (Figure 5).

### Functional profile

In addition to a taxonomic profile, shotgun sequencing provides information regarding samples' functional profile. The relative abundance of the 20 most abundant functional features is shown in Figure 7. Relative abundance of some functional features tended to be numerically increased in samples from BRD calves compared to non-BRD calves (Amino Acids and derivatives:  $P = 0.87$ ; Carbohydrates:  $P = 0.84$ ; Cell division and cell cycle:  $P = 0.95$ ; Cell wall

and capsule:  $P = 0.99$ ; Clustering-based subsystems:  $P = 0.61$ ; Cofactors, vitamins, prosthetic groups, pigments:  $P = 0.88$ ; DNA metabolism:  $P = 0.97$ ; Dormancy and sporulation:  $P = 0.17$ ; Fatty acids and isoprenoids:  $P = 0.94$ ; Iron acquisition and metabolism:  $P = 0.90$ ; Membrane transport:  $P = 0.95$ ; Metabolism and aromatic compounds:  $P = 0.09$ ; Miscellaneous:  $P = 0.88$ ; Motility and chemotaxis:  $P = 0.83$ ; Nitrogen metabolism:  $P = 0.93$ ; Nucleosides and nucleotides:  $P = 0.87$ ; Phages, prophages, transposable elements and plasmids:  $P = 0.88$ ; Phosphorus metabolism:  $P = 0.67$ ; Photosynthesis:  $P = 0.82$ ; Potassium metabolism:  $P = 0.99$ ; Protein metabolism:  $P = 0.73$ ; RNA metabolism:  $P = 0.81$ ; Regulation and cell signaling:  $P = 0.58$ ; Secondary metabolism:  $P = 0.93$ ; Stress metabolism:  $P = 0.76$ ; Sulfur metabolism:  $P = 0.93$ ; Virulence and Disease and Defense factors:  $P = 0.69$ ).

Analysis of virulence and disease and defense factors revealed numeric differences in resistance to antibiotics and toxic compounds features ( $P = 0.12$ ) between healthy calves and calves that developed BRD. Results from this analysis are presented in Figure 8. Bacteriocins was only detected in BRD-14d calves. Adhesion factors were more abundant in healthy-28d calves compared to the other groups ( $P = 0.67$ ) (Figure 8).

The resistance to antibiotics and toxic compounds analysis revealed differences between BRD-14d and healthy-14d calves in cobalt-zinc-cadmium resistance (Figure 9). These differences tended to be statistically significant ( $P = 0.07$ ). The relative abundance of multidrug resistance efflux pumps was numerically increased in BRD-14d ( $0.11 \pm 0.08$ ) and BRD-28d ( $0.10 \pm 0.08$ ) comparing to healthy calves ( $P = 0.57$ ). Resistance to fluoroquinolones was numerically increased in healthy-28d calves ( $0.38 \pm 0.13$ ) and BRD-28d ( $0.29 \pm 0.16$ ) ( $P = 0.57$ ). Methicilin-resistant *Staphylococcus* ( $P = 0.29$ ) and copper homeostasis features ( $P = 0.66$ ) were both numerically increased in BRD-28d ( $0.19 \pm 0.16$ ;  $0.04 \pm 0.04$ , respectively). Beta-lactamase ( $P = 0.55$ ) was only detected in healthy-14d calves ( $0.01 \pm 0.01$ ). Cadmium resistance was numerically increased in BRD-28d calves ( $0.08 \pm 0.08$ ) compared to the other groups ( $P = 0.25$ ) (Figure 9).

DISCUSSION

In an effort to better understand the upper respiratory tract metagenome in dairy calves, we used shotgun sequencing of DNP samples and characterized the differences between healthy animals at 14 and 28 days of life and animals that developed BRD between these two time points. These two time points were selected mainly because all BRD cases in the Lima et al. (2016) study were diagnosed between the 14<sup>th</sup> and the 28<sup>th</sup> day of life. Our data revealed a remarkable complexity in the bacterial ecology of both healthy and BRD affected calves. *Moraxella catarrhalis*, a species well known to be associated with upper respiratory tract disease in humans, was detected in all groups, particularly in BRD calves, although this difference was not statistically significant. Upon functional profile analysis, we observed greater abundance of Cobalt-Zinc-Cadmium resistant genes in BRD-14d samples, which could indicate difficulties in treatment of these animals, given that the presence of these genes has been associated with increased resistance to antibiotics.

It should be noted that our study could have been more informative had we been able to obtain bronchoalveolar lavage samples (at least at the second sampling time point) which would have allowed us to study the lung metagenome in addition to the upper respiratory tract metagenome. However, our study was conducted on a commercial dairy farm and it was not possible for us to secure the owner's consent for such a sampling approach. The small number of enrolled calves represents another limitation of our study. Although shotgun metagenomic studies are commonly conducted on small number of subjects (as the cost of conducting such studies remains high) this limitation should be acknowledged and results presented here should be interpreted with caution. It should also be noted that our study was conducted on a single farm; a larger scale, multi-farm study would have potentially been more informative. Sampling time points were fixed (14 and 28 days of life) and dictated by the Lima et al. 2016 study design. This

represents another limitation of our study since calves developed BRD and were treated with antibiotics at different time points (between 14 and 28 days of life).

Calves that were diagnosed with BRD in our study received antibiotic therapy. Systemic antibiotic therapy in calves should impact the microbiome of the URT. Therefore the post treatment upper respiratory tract microbiome is likely a reflection of both the micro-organisms involved in the disease process and the impact of antimicrobial treatment. We have recently shown that parenteral administration of antibiotics has a temporal effect on the neonatal calf's fecal microbiome and this should also be the case for the upper respiratory tract microbiome (Oultram et al. 2015). Our study was conducted on a commercial dairy farm and farm protocols could not be modified solely for the purpose of the study. Additionally, BRD is commonly caused by bacterial infection and neglecting to treat sick calves with the proper antibiotic therapy could be considered inhumane and may not have been approved by the Cornell Institutional Animal Care and Use Committee. Differences between samples obtained at different time points could also be due to the evolution of the nasopharyngeal microbiota. Timsit et al. (2016) recently reported that the nasopharyngeal microbiota of beef cattle underwent a profound evolution from weaning until arrival at a feedlot and from arrival to day 40.

Analysis at phylum-level detected 22 phyla, of which *Proteobacteria*, *Firmicutes*, *Actinobacteria*, *Bacteroidetes* and *Tenericutes* were the most abundant. Our results are in agreement with those by Holman et al. (2015a) who, studying the nasopharyngeal microbiome of healthy and BRD calves, detected 22 phyla of which *Proteobacteria* and *Firmicutes* were the major ones. Studies in pigs (Lowe et al., 2011) and children (Bogaert et al., 2011) revealed similar results at the phylum level. Conversely, the phylum *Tenericutes* was dominating the nasopharyngeal microbiota of beef cattle from weaning to 40 days after their arrival at a feedlot (Timsit et al., 2016).

Analysis at the genus-level revealed 20 major genera that belonged to the aforementioned five phyla. Among them, significant differences were detected in *Pseudomonas* spp. relative



abundance between different groups of calves. *Pseudomonas fluorescens* was the species responsible for this significant difference being more abundant in BRD-14d calves. Reduction in its abundance in BRD-28d calves could be associated with antibiotic treatment. *Pseudomonas fluorescens* has been associated with the production of an antifungal lipopeptide for biocontrol of plant pathogenic fungus (Nielsen et al., 2000). In humans, urinary tract infections (Von Graevenitz and Weinstein, 1971), bacteremia in oncology patients (Hsueh et al., 1998) and a possible role in the intestinal mucosal damage in Crohn's disease patients (Wei et al., 2002; Scales et al., 2014) have all been attributed to *Pseudomonas fluorescens*. Although *Pseudomonas fluorescens* has been identified by culture-independent and culture-dependent techniques in bronchoalveolar lavage fluid (Bahrani-Mougeot et al., 2007) and tracheal aspirates (Redding and McWalter, 1980) in patients with pneumonia, its role in respiratory disease remains unclear. In ruminants, *Pseudomonas fluorescens* has not been reported as a causative agent of any infections.

*Mannheimia haemolytica*, *Mycoplasma bovis*, *Histophilus somni* and *Pasteurella multocida* are the most commonly reported bacteria in pneumonia cases in cattle (Rice et al., 2007; Griffin et al., 2010). In the present study *Mannheimia haemolytica* was the most abundant bacterium in all groups and was detected in most of the studied calves. Its relative abundance was increased in both group of calves, particularly in the healthy-28d one. *Mannheimia haemolytica* is an upper respiratory tract commensal that can become pathogenic under certain circumstances (Griffin et al., 2010). Several studies have reported the presence of *Mannheimia haemolytica* in the respiratory tract of both healthy and BRD calves, most of which observed an association between this bacterium and pneumonia (Angen et al., 2009; Francoz et al., 2015; Holman et al., 2015a). The analysis of *Mannheimia haemolytica* strains showed the presence of serotype A2 in all groups. This is the predominant strain detected in healthy calves (Rice et al., 2007) and it is associated with pneumonia in sheep (Lawrence et al., 2010). On the other hand, pneumonia in cattle is highly associated with serotypes A1 and A6 (Griffin, 2010). Interestingly, we detected numeric differences



in *Mannheimia haemolytica* serotype A1 str. PHL 213 relative abundance between healthy and BRD calves, which is in accordance with previous research (Purdy et al., 1997; Al-Ghamdi et al., 2000; Griffin et al., 2010).

Despite being commonly reported as a causative agent of pneumonia in calves (Kusiluka et al., 2000; Gagea et al., 2006), we could only detect *Mycoplasma bovis* in very low relative abundances. *Mycoplasma bovis* sequences were detected in one out of six BRD-14d calves, one out of six BRD-28d calves, and four out of ten healthy 28d calves. *Mycoplasma bovis* sequences were absent from all the healthy-14d calves. The rather low relative abundance of *Mycoplasma bovis* observed and the presence of other pneumonia-associated relevant bacteria, support the hypothesis that *Mycoplasma bovis*, is an opportunistic pathogen (Rosendal and Martin, 1986) and part of the normal upper respiratory tract microbiome of calves.

*Histophilus somni* is a commensal microorganism that belongs to the nasopharyngeal microbiome of calves (Griffin et al., 2010) and like other bacteria that reside in the upper respiratory tract, becomes pathogenic after changes in the upper respiratory tract environment. In the present study, *Histophilus somni* was less abundant than *Mannheimia haemolytica*, *Mycoplasma bovis*, and *Pasteurella multocida*.

*Pasteurella multocida* was one of the 20 most abundant bacteria detected in the deep nasal swabs samples. It was detected in both healthy and sick groups, although it was not identified in all samples. *Pasteurella multocida* was detected in BRD calves in slightly higher abundance; particularly on 14 days of life; however no statistically significant differences between groups were detected. Several studies detected *Pasteurella multocida* in the respiratory tract of calves, using culture-dependent and culture-independent techniques; in most of them, this bacterium was highly prevalent (Angen et al., 2009; Jamali et al., 2014; Holman et al., 2015b).

*Moraxella catarrhalis* is a well-known Gram-negative pathogen associated with respiratory disease in humans (Verduin et al., 2002; Goldstein et al., 2009), particularly in children (Sy and Robinson, 2010). Although it is described as an exclusive pathogen of the upper respiratory tract of humans (Goldstein et al., 2009), *Moraxella catarrhalis* was isolated from lungs of BRD calves and its presence was also demonstrated in lung lesions from the same calves by immunohistology in the study conducted by Nagai et al., (1995). Recently, *Moraxella* spp. was detected in the nasopharyngeal tract of calves in a feedlot (Holman et al., 2015a), however, the species was not determined and the health status of the calves was not reported.

*Actinobacillus pleuropneumoniae*, *Mycoplasma hyopneumoniae* and *Haemophilus parasuis* were among the 20 most abundant bacteria detected in DNP swab samples. These species are well-known pathogens associated with swine respiratory disease (Bossé et al., 2002; Nedbalcova et al., 2006; Sibila et al., 2009). Although *Actinobacillus pleuropneumoniae* is considered an exclusive pathogen of the swine respiratory tract, this species has previously been isolated from cattle, deer and lambs (Desrosiers et al., 1998). The potential role of these fastidious bacteria in BRD may warrant further investigation.

Functional profiling showed numeric differences between healthy calves and calves that developed BRD, the latter having increased abundances of resistance to antibiotics and toxic compounds features. Cadmium, zinc and cobalt resistance is observed in bacteria such as *Staphylococcus aureus* and *Escherichia coli* and is related to proteins such as *cadCA cadB*, *czcCBA* and *cnr*, depending on the bacteria (Nies, 1992). Five mechanisms of metal-resistance are described (Choudhury and Srivastavara, 2001): exclusion of metal by permeable barrier, exclusion by active transport, intra and extracellular physical sequestration and transformation and detoxification. Studies reported heavy metals as a source of co-selection of antibiotic resistance (Pettibone et al., 1996; Stepanauskas et al., 2005; Seiler and Berendonk, 2012). Co-selection occurs due to physiological (cross-resistance) and genetic (co-resistance) mechanisms. The first provides

resistance to more than one antibiotic agent (drugs and heavy metal) using mechanisms such as Multi Drug Efflux Pumps (Baker-Austin et al., 2006; Martinez et al., 2009) and the second one is related to two or more genetically linked resistance genes (Chapman, 2003). In the present study, genes related to heavy metal resistance were increased in BRD calves compared with healthy calves and this could imply difficulties in the treatment of diseased calves, given that studies have highlighted the importance of co-selection and heavy metal resistance for antibiotic resistance (Chapman, 2003). Genes associated with antibiotic resistance were also more abundant in samples obtained from BRD calves.

## CONCLUSION

In this particular study, *Mannheimia haemolytica* and *Pasteurella multocida* were part of the 20 most abundant bacteria found in healthy and BRD calves. *Moraxella catarrhalis*, an important bacterium for human pneumonia, was detected in both groups, particularly in BRD calves. The importance of *Moraxella catarrhalis* for calves' respiratory disease remains elusive. Cobalt-Zinc-Cadmium resistant genes observed mostly in BRD calves might be associated with difficulties in the treatment using antibiotics.

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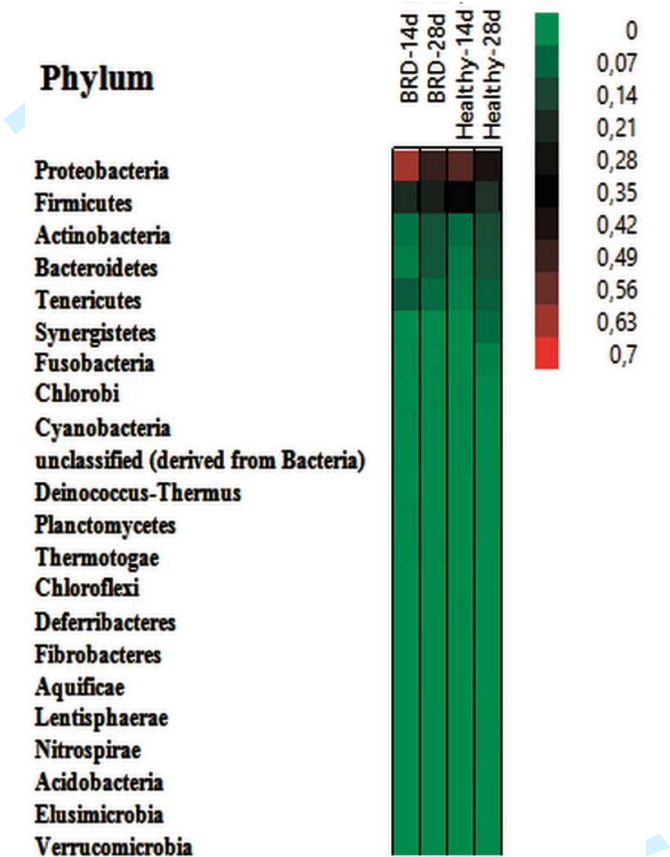
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557

558 **Figure 1.** Heat map demonstrating the relative abundances of different phyla in deep  
559 nasopharyngeal swab samples obtained from: healthy calves at 14 days of life (healthy-14d); calves  
560 that developed respiratory disease between 14 and 26 days of life (samples obtained at 14 days of  
561 life, BRD-14d); healthy calves at 28 days of life (healthy-28d); and calves that developed BRD  
562 between 14 and 26 days of life (samples obtained at 28 days of life, BRD-28d).

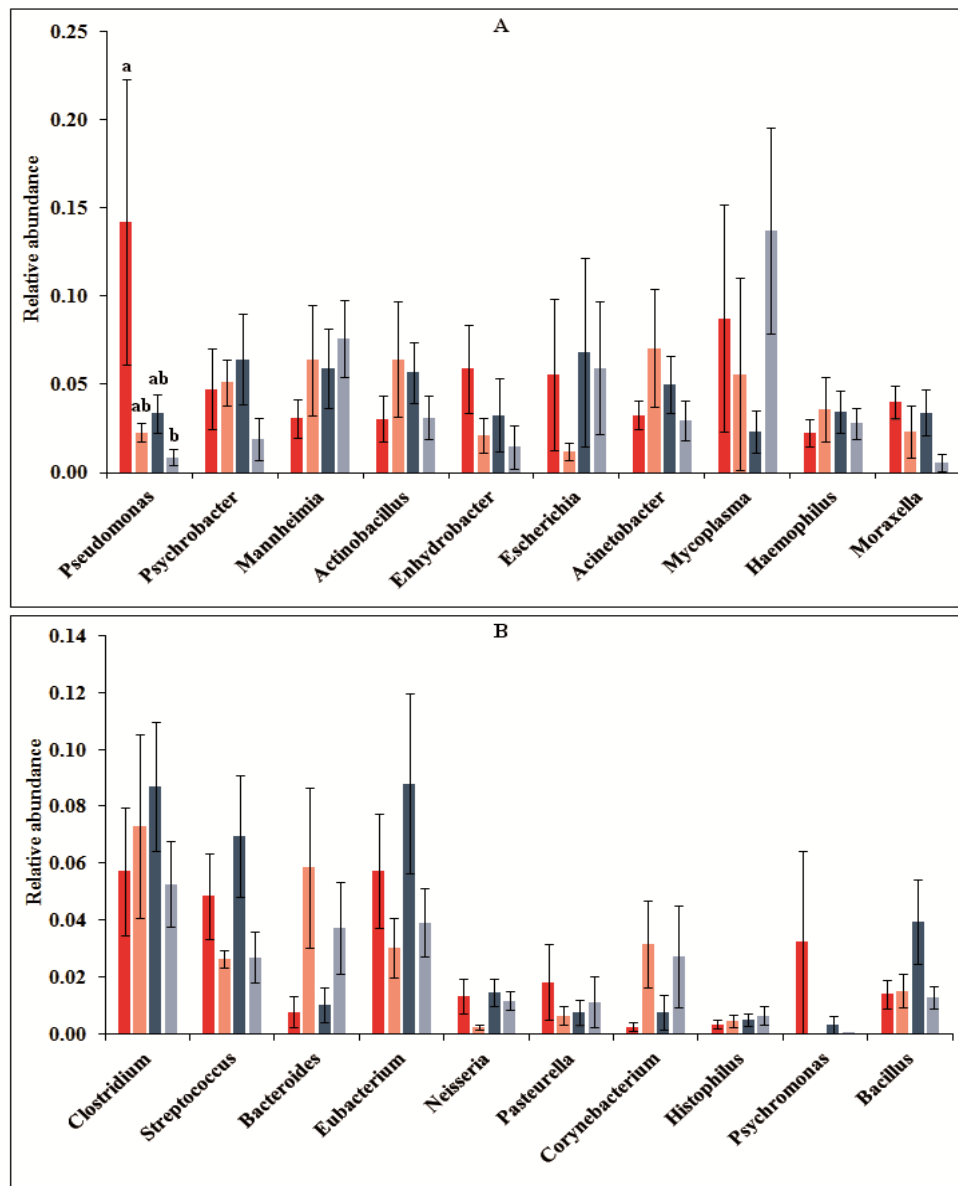
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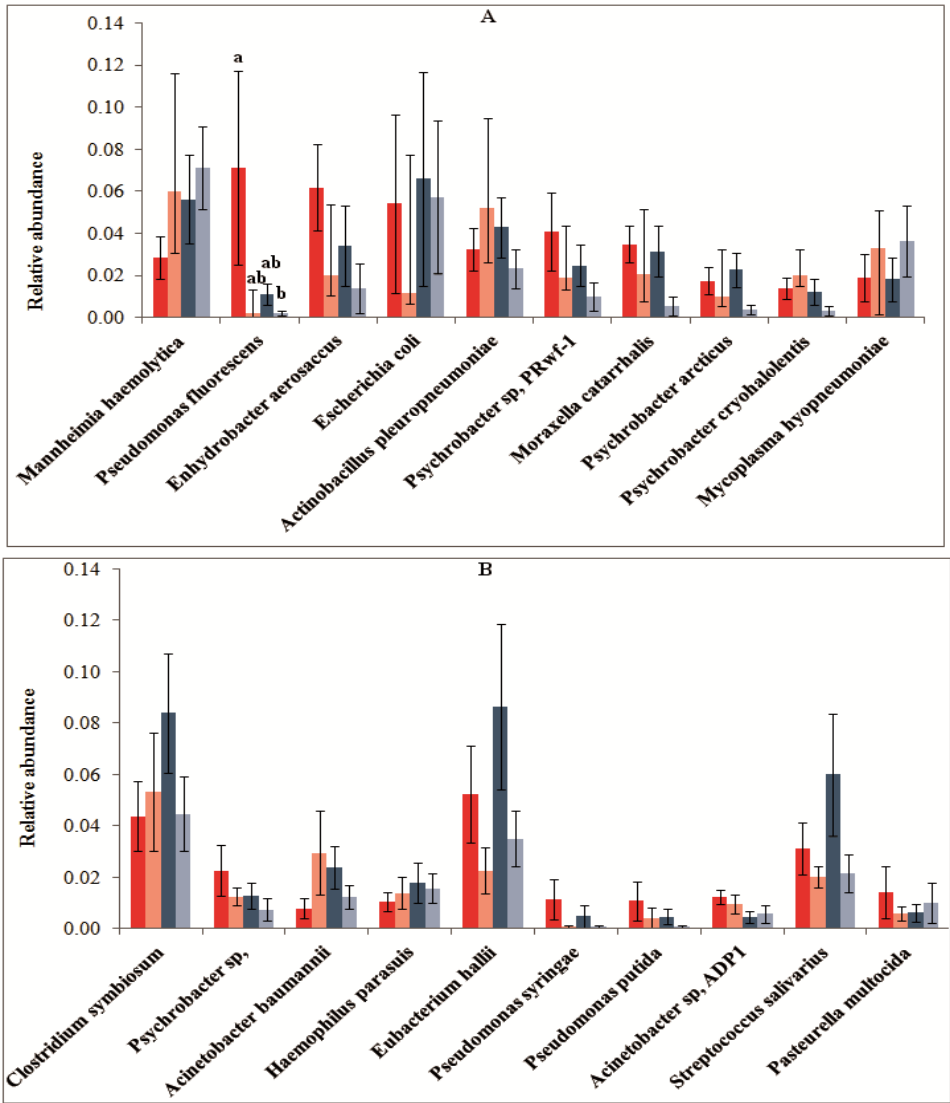
565 **Figure 2.** Relative abundance of the 20 most abundant genera (the ten most abundant of them are  
 566 presented in subfigure A with the rest presented in subfigure B) in deep nasopharyngeal swab  
 567 samples obtained from healthy calves (blue) and calves that developed respiratory disease (red) at  
 568 day 14 (dark colors) and day 28 (light colors) of life. Error bars represent standard error of the  
 569 mean. Different letters indicate Tukey-adjusted significant difference between groups ( $P < 0.05$ ).



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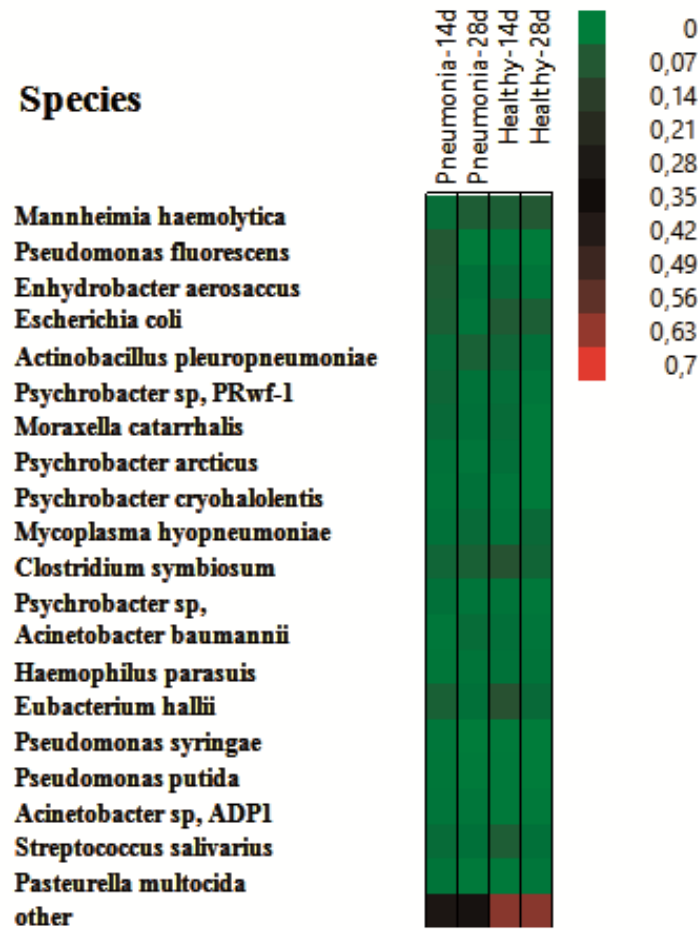
572 **Figure 3.** Relative abundance of the 20 **most abundant** species (the ten **most abundant** of them are  
573 presented in subfigure A with the rest presented in subfigure B) in deep nasopharyngeal swab  
574 samples obtained from healthy calves (blue) and calves that developed respiratory disease (red) at  
575 day 14 (dark colors) and day 28 (light colors) of life. Error bars represent standard error of the  
576 mean. Different letters indicate Tukey-adjusted significant difference between groups ( $P < 0.05$ ).



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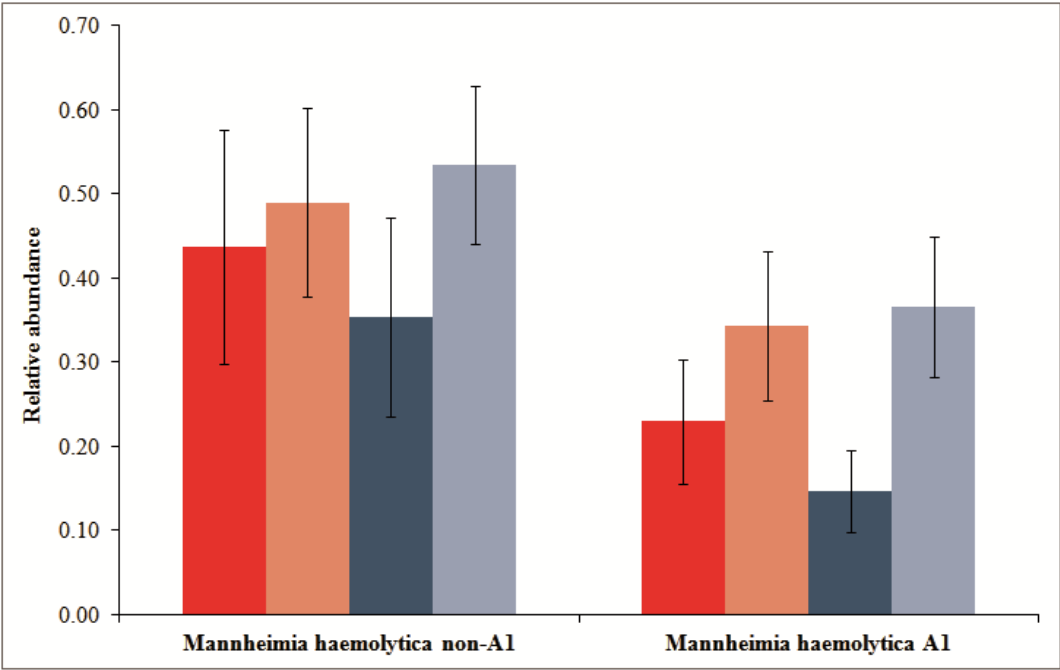
579 **Figure 4.** Heat map demonstrating the relative abundances of different species in deep  
580 nasopharyngeal swab samples obtained from: healthy calves at 14 days of life (healthy-14d); calves  
581 that developed respiratory disease between 14 and 26 days of life (samples obtained at 14 days of  
582 life, BRD-14d); healthy calves at 28 days of life (healthy-28d); and calves that developed BRD  
583 between 14 and 26 days of life (samples obtained at 28 days of life, BRD-28d).



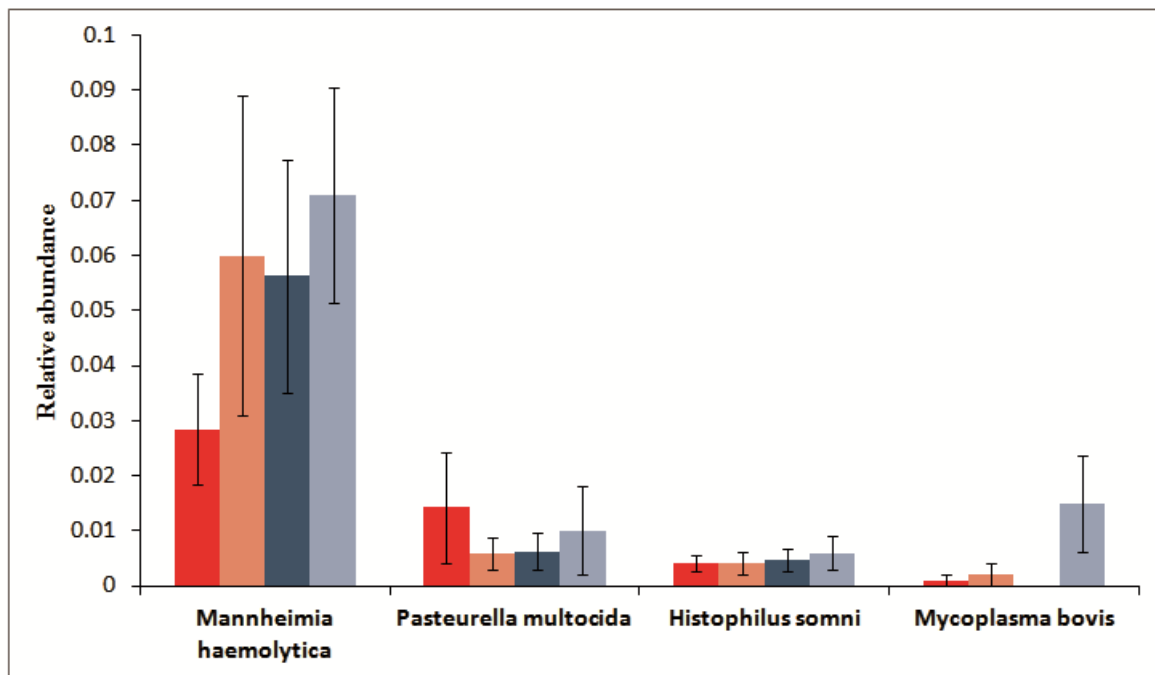
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**Figure 5.** Relative abundance of *Mannheimia haemolytica* non-A1 and *Mannheimia haemolytica* A1 in deep nasopharyngeal swab samples obtained from healthy calves (blue) and calves that developed respiratory disease (red) at day 14 (dark colors) and day 28 (light colors) of life. Error bars represent standard error of the mean. Statistically significant differences were not observed.

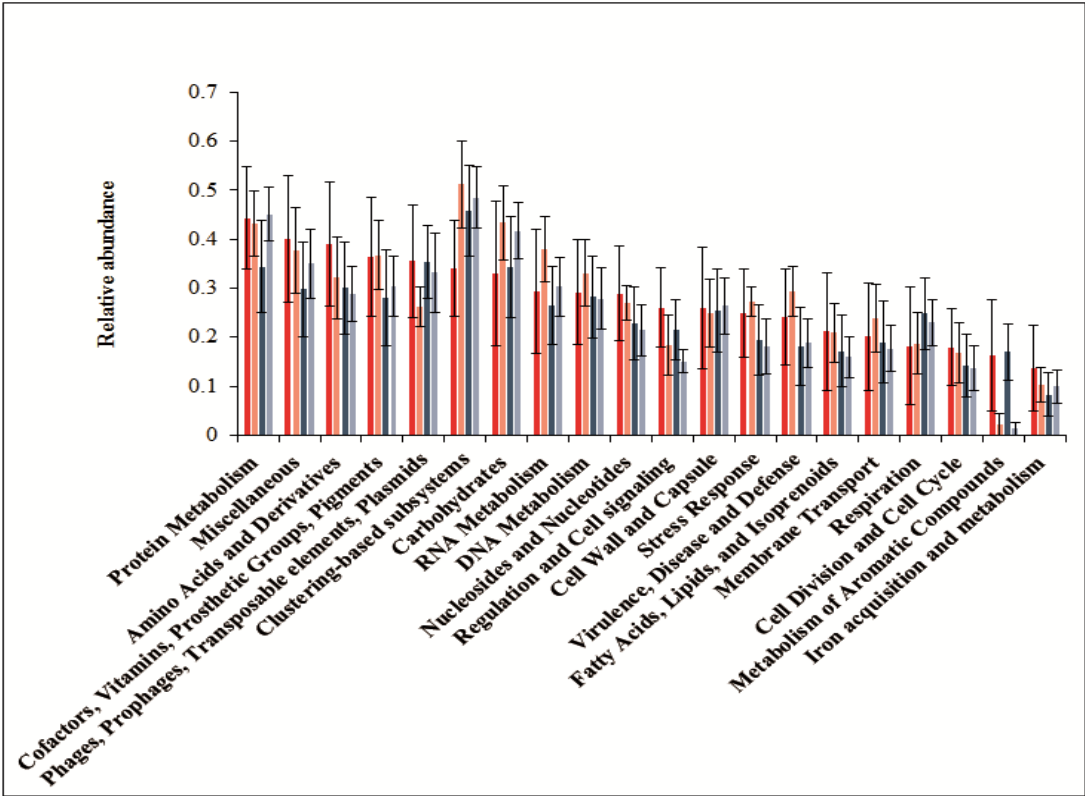


**Figure 6.** Relative abundance of the most common bacteria related to BRD in deep nasopharyngeal swab samples obtained from healthy calves (blue) and calves that developed respiratory disease (red) at day 14 (dark colors) and day 28 (light colors) of life. Error bars represent standard error of the mean. Statistically significant differences were not observed.



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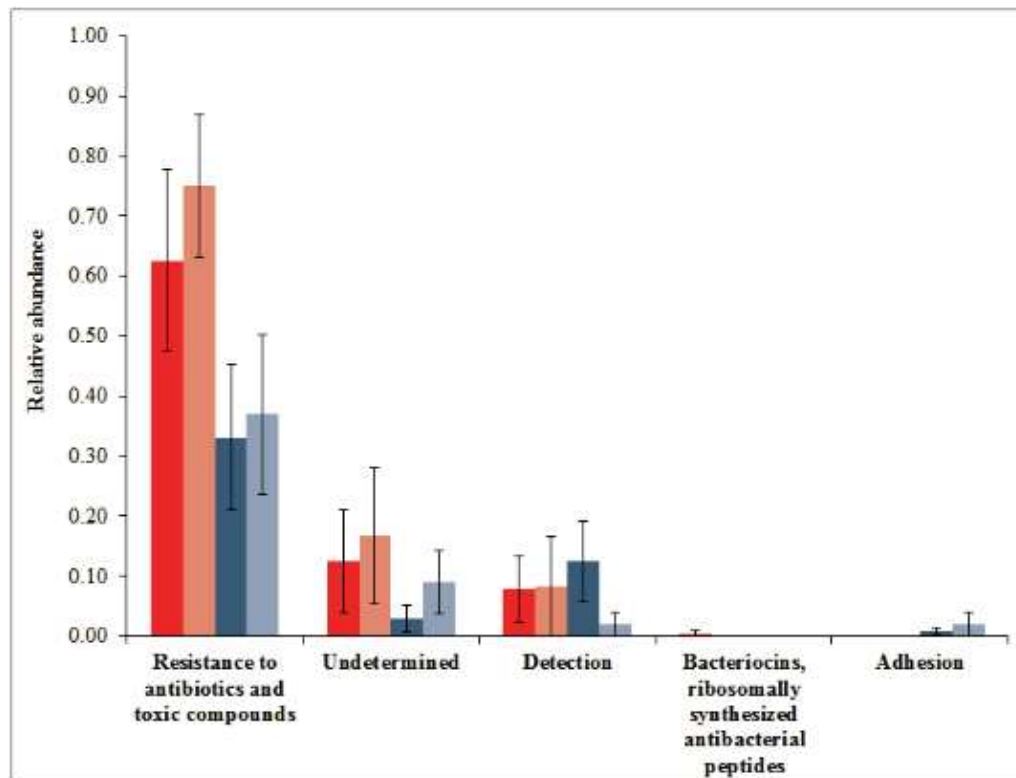
603 **Figure 7.** Relative abundance of the 20 most **abundant** functional profile features in deep  
604 nasopharyngeal swab samples obtained from healthy (blue) and BRD calves (red) on day 14 (dark  
605 colors) and day 28 (light colors) of their life. Error bars represent standard error of the mean.  
606 Statistically significant differences were not observed.



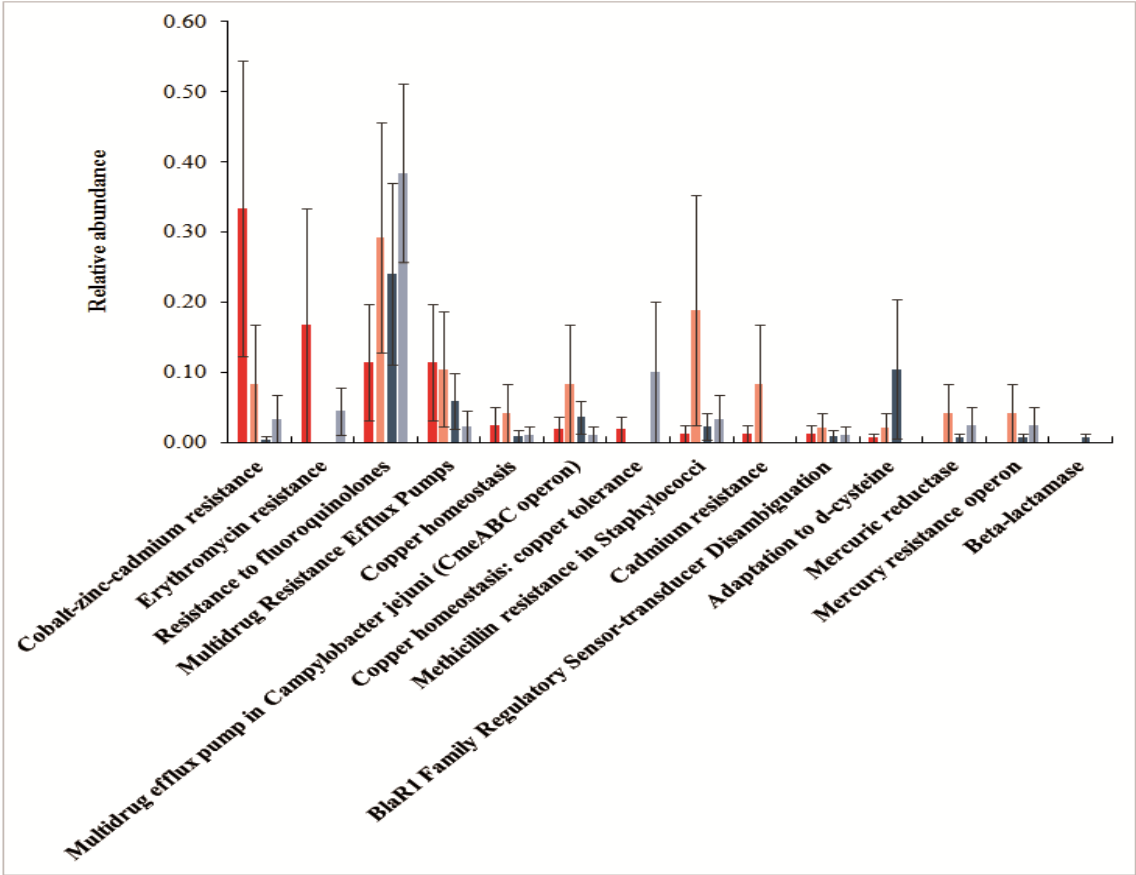
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**Figure 8.** Normalized abundance distribution of virulence and disease and defense factors in deep nasopharyngeal swab samples obtained from healthy (blue) and BRD (red) calves at day 14 (dark colors) and day 28 (light colors) of their life. Error bars represent standard error of the mean. Statistically significant differences were not observed.



**Figure 9.** Normalized abundance distribution of resistance to antibiotics and toxic compounds features in deep nasopharyngeal swab samples obtained from healthy (blue) and BRD (red) calves at day 14 (dark colors) and day 28 (light colors) of their life. Error bars represent standard error of the mean. Statistically significant differences were not observed.





**Table 1.** Proportion of calves with a positive detection of BRD bacterial pathogens according to their health status (healthy and BRD calves) and sampling time (14<sup>th</sup> and 28<sup>th</sup> day).

Pneumonic pathogens	BRD-14d	BRD-28	Healthy-14d	Healthy-28d	Total
	N (%)	N (%)	N (%)	N (%)	N (%)
<i>Mannheimia haemolytica</i>	4 (66.7)	5 (83.3)	7 (70.0)	9 (90.0)	25 (78.12)
<i>Histophilus somni</i>	4 (66.7)	4 (66.7)	4 (40.0)	4 (40.0)	16 (50.0)
<i>Pasteurella multocida</i>	4 (66.7)	3 (50.0)	4 (40.0)	4 (40.0)	15 (46.69)
<i>Mycoplasma bovis</i>	1 (16.7)	1 (16.7)	0 (00.0)	4 (40.0)	6 (18.75)